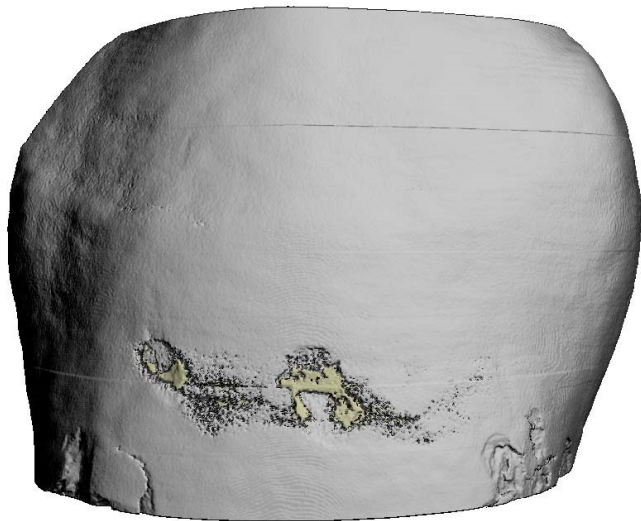


**MICROBIOLOGICAL, CLINICAL AND 3-DIMENSIONAL FEATURES OF
WHITE SPOT LESIONS IN PERMANENT TEETH**

Lino Thorlakson

OSLO 2013



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*Series of dissertations submitted to the
Faculty of Dentistry, University of Oslo*

ISBN 978-82-91757-88-9

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Department of Oral Biology

Faculty of Dentistry

The University of Oslo, Oslo

Norway



Department of Microbiology

The Forsyth Institute, Cambridge, MA

USA



Academic Dissertation

This thesis will be defended for the degree Philosophiae Doctor (Ph.D.) at the Faculty of

Dentistry, University of Oslo, Oslo,

Norway

Adjudication committee:

Ann Progulske-Fox, PhD

Professor and Director

Department of Oral Biology

University of Florida

Gainesville, FL, USA

Theodore Eliades, DDS, MS, Dr Med Sci, PhD, FRSC, FIMMM

Professor and Director

Department of Orthodontics and Paediatric Dentistry

Center of Dental Medicine, University of Zurich

Plattenstrasse 11, CH-8032 Zurich, Switzerland

Tiril Willumsen, PhD

Professor

Department of Pediatric Dentistry and Behavioural Science

PB 1109 Blindern 0317 Oslo, Norway

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1. ACKNOWLEDGMENTS

The work with this thesis was carried out at the Department of Oral Biology, Faculty of Dentistry, The University of Oslo, Norway, and the Department of Microbiology, Forsyth Institute, Boston, USA and the Institute of Applied Structure and Microanalysis, Johannes Gutenberg University, Mainz, Germany.

This thesis could not have been completed without the financial support from the Faculty of Dentistry, University of Oslo and from grants provided by the University of Oslo (Småforsk and MLS^{UIO}).

I am deeply grateful to both of my principle supervisors Prof. Bjørn Øgaard and Prof. Ingar Olsen for opening the doors of science and research to my life. I wish to thank them for sharing their vast knowledge of microbiology, cariology and orthodontics with me, for their guidance, enthusiasm and dedication to help me. Their genuine kindness and desire to help has been a tremendous asset. I would also like to thank Prof. Bjørn Øgaard for allowing me to take plaque samples from patients in his private practice.

This thesis would have also not been possible were it not for the generous help provided by orthodontist Jon-Olav Aabakken and the secretaries at his clinic in Hamar. I collected the majority of samples needed for this thesis at his clinic. Jon-Olav Aabakken and his secretaries went out of their way in order to help me find enough patients for this thesis. I am very grateful to the students at the Department of Orthodontics, Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo for providing me with additional patients needed for this thesis.

I would like to thank Prof. Bruce J. Paster and his research group at the Forsyth Institute for their help with the microarray analysis and allowing me the opportunity to use their facilities for my research. At the Department of Oral Biology, I received a great deal of support and guidance from Dr. scient. Anne K. Kristoffersen in the laboratory. I am very grateful for her help and kindness. My deepest gratitude goes to my PhD colleague Ibrahimu Mdala for his advice regarding the statistical analysis of the data. I am very grateful to Håkon Størmer and the Photo Department for providing me with the high quality images I needed for my research. I appreciate all the technical and practical assistance I received from everyone from the faculty's administration, including Per A. Omdal, Gry Olsen, Åslaug S. Brynildsen and Tor Henry Wold.

I wish to thank my parents for giving me a privileged upbringing, introducing me to the fields of science and research from an early age, and allowing me to develop an interest and passion for the pursuit for knowledge. My deepest gratitude also to my understanding and supporting wife, who is in the same time working on her own doctoral thesis.

2. PREFACE AND LIST OF PAPERS

The following papers (I-III) are submitted in fulfillment of the requirements for the degree Philosophiae Doctor (Ph.D.) at the Faculty of Dentistry, University of Oslo, Oslo, Norway.

This thesis is based on experimental work carried out at The Forsyth Institute (Boston, USA) and the Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo (Oslo, Norway).

The papers will be referred to in the present summary by their Roman numerals.

PAPER I

Torlakovic L, Klepac-Ceraj V, Ogaard B, Cotton SL, Paster BJ, Olsen I.

Microbial community succession on developing lesions on human enamel.

J Oral Microbiol. 2012;4. doi: 10.3402/jom.v4i0.16125. Epub 2012 Mar 14.

PAPER II

Torlakovic L., Paster B.J., Øgaard B., Olsen I.

Changes in the supragingival microbiota surrounding brackets of upper central incisors during orthodontic treatment.

Acta Odontol Scand, 2013; in press.

PAPER III

Torlakovic L., Olsen I., Petzold C., Tiainen H., Øgaard B.

Clinical color intensity of white spot lesions (WSLs) may be a better predictor of enamel demineralization depth than traditional WSL clinical grading.

Am J Orthod Dentofacial Orthop. 2012;142(2):191-8. doi: 10.1016/j.ajodo.2012.03.025.

3. ABBREVIATIONS

16S rRNA	16 Svedberg ribosomal RNA
Caries	Dental caries
CLSM	Confocal laser scanning microscopy
CT	Computed tomography
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
EM	Electron microscopy
FAME	Fatty acid methyl ester
FISH	Fluorescent <i>in situ</i> hybridization
GC clamp	A stretch of GC-rich sequences used in DGGE
GC ratio	The ratio of the GC to AT base pairs in DNA
HOMIM	Human Oral Microbial Identification Microarray
HSP	Heat shock proteins
LF	Laser Fluorescence
MLS ^{UIO}	Molecular Life Sciences at the University of Oslo
MLST	Multi Locus Sequence Typing
OTUs	Operational taxonomic units. Defined using a 97% sequence similarity cutoff.
PCR	Polymerase chain reaction
QLF	Quantitative Light-induced Fluorescence
RNA	Ribonucleic acid
TMR	Transverse microradiography
WSL	White spot lesion

4. GENERAL INTRODUCTION

4.1 ETIOLOGY OF DENTAL CARIES

One of the earliest attempts to elucidate the etiology of dental caries comes from a Sumerian text from 5,000 BC, describing a "tooth worm" as the cause of caries [1]. Evidence of this idea has also been found in countries such as India, Egypt, Japan, and China [2]. During the European Age of Enlightenment (eighteenth century AD), the belief that a "tooth worm" caused caries was no longer accepted in the European medical community [3]. The father of modern dentistry, Pierre Fauchard was one of the first to reject the "tooth worm" theory and noticed that sugar was harmful to the teeth and gingiva.

Willoughby D. Miller was an American dentist and the first oral microbiologist. In the 1890s, he conducted a series of studies that led him to suggest an explanation for dental caries that was influential for current theories. He found that bacteria inhabited the mouth and that they produced acids which dissolved tooth structures in the presence of fermentable carbohydrates [4]. This explanation is known as the 'chemoparasitic caries theory'. Miller's research, along with the research on dental plaque by G.V. Black and J.L. Williams, served as foundations for the etiology of the caries model [2]. In the 1970's the 'specific plaque hypothesis' [5] emerged, which addressed dental caries as an infectious disease caused by *Streptococcus mutans*. Later on the 'ecological plaque hypothesis', and its many variations, [4, 6, 7, 8 and 9] have stressed the importance of shifts in the proportions of bacterial species within a mixed plaque population, rather than a single bacterium for the etiology of caries. Recent advances in biotechnology have allowed us to explore the microbiological community of dental plaque in more detail using high-throughput genetic methods.

Molecular methods based on PCR amplification of total DNA from plaque samples followed by sequencing of 16S rDNA and 16S rDNA-based microarray analysis have led to an expanded list of caries-associated bacterial genera such as *Actinomyces*, *Abiotrophia*, *Atopobison*, *Bifidobacterium*, *Lactobacillus*, *Atopobium*, *Scardovia*, *Olsenella*, *Pseudoramibacter*, *Selenomonas* and *Veillonella* [10, 11, 12, 13, 14 and 15]. Furthermore, several studies support the view that caries can occasionally develop in the absence of *S. mutans* [14, 16 and 17].

The recognition that many bacterial species may be involved in the development of caries does not mean that *S. mutans* does not play an important role. In fact a systematic review found 2,538 articles on the topic supporting the role of *S. mutans* in the etiology of caries [18]. *Lactobacillus* has also been one of the main bacteria associated with dental caries and has been studied intensively since the early twentieth century [19]. While it is widely accepted that *S. mutans* and *Lactobacillus* can be important culprits of dental caries, it is also clear that bacteria other than *S. mutans* and *Lactobacillus* play important roles in the etiology of caries development. Therefore *S. mutans* and *Lactobacillus* should not be our sole focus, and more research is needed to elucidate the complexity of dental caries. We now know that independent of the species, in order to cause caries the bacteria must be acidogenic (acid producing) and aciduric (acid tolerant). In addition to the bacteria, caries is a multifactorial disease involving interactions among the diet, saliva and properties of the tooth surface.

4.2 THE ENAMEL LESION

The white spot lesion (WSL) is an enamel lesion. G.V. Black was the first to study the time it took for dental plaque at areas of plaque stagnation to cause enamel lesions. In his experiments, published in 1918, he allowed dental plaque undisturbed by mechanical forces to accumulate for days and weeks. After just 1 week, even though no clinical signs were present, histologically one could see signs of increased enamel porosity. After 2 weeks, WSLs were clinically visible after careful air drying. Histologically a subsurface lesion formed, with mineral loss most apparent under the outer enamel layer. After 3 and 4 weeks, air drying was not needed to see the WSL. The lesions were more irregular with the development of pits of Tome's processes and focal holes.

In dental plaque microorganisms adhere to each other and/or to the pellicle of the tooth surface creating what is known as a biofilm. Plaque is found naturally on tooth surfaces and is a part of the host defenses. However, the plaque can accumulate to such levels and changes in its microbial composition that it becomes incompatible with oral health. The plaque overlying the buccal/labial WSLs that can develop during treatment with fixed orthodontics is smooth surface plaque. The geometry of teeth creates different ecological niches where different species of bacteria can thrive. Therefore, the bacterial composition of smooth surface plaque will vary compared to that of gingival, subgingival, proximal and fissure plaque.

The amount of plaque present on a tooth surface and its period of accumulation influence the composition of the microbiota within the plaque. A newly cleaned tooth surface is rapidly covered with a glycoprotein layer called the acquired pellicle which consists of

mucins, albumin, proline-rich proteins, amylase and lysozyme. Shortly after, gram positive bacteria follow, such as *S. mutans* and *Streptococcus. sanguinis*. The pellicle contains proteins that these gram positive bacteria exploit for attachment [20]. The bacterial load in the plaque now starts increasing due to cell division of the gram positive 'primary colonizers' and due to the attachment of new species including gram negative species such as *Fusobacterium nucleatum*, *Prevotella intermedia* and *Capnocytophaga* spp. (which can be found after 1 to 3 days). After 7 days other species appear, such as the gram negative *Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus*, *Porphyromonas gingivalis*, *Eikenella corrodens*, and *Treponema* spp.

WSLs are incipient caries lesions and precursors of frank enamel caries. At the dental plaque – enamel interface the lesion results from repeated rounds of demineralization and remineralization. The manner in which the enamel progresses from sound surface to WSL was first described by G.V. Black in his textbook of 1908. He created a protective area on a tooth surface where plaque was allowed to accumulate undisturbed. Clinically he found that after one week no macroscopic changes could be seen even after air drying. After two weeks whitish lesions could be detected on the enamel surface after air drying. After 3-4 weeks larger irregularities developed and the enamel lesion could be detected without air-drying. Pioneers of microscopic examination of the enamel lesion were G.V. Black and V. Andresen. In 1921 orthodontist V. Andresen performed controlled demineralizations on teeth isolated with a rubber dam that were destined for extraction due to orthodontic treatment. After exposing the teeth to an acidic compound the teeth were extracted and the lesions analyzed microscopically. Other well known, more advanced microscopic studies of enamel were performed in the 1980's [21, 22, 23 and 24]. After one week of plaque

retention, accumulation and maturation the enamel ultrastructure showed signs of dissolution of the outer enamel surface (wider intercrystalline spaces and increased enamel porosity). After 2 weeks an increased porosity was observed, but now due to dissolution of mineral below the outer surface. In addition, a mineral-rich outer layer could be seen. These phenomena create a sub-surface lesion. The exact reason for the formation of the outer layer is difficult to explain. Evidence points to fluoride as the main inhibiting agent of demineralization, however, proteins may also play a role. Arends and Christoffersen [25] have created a kinetic mathematical model that attempts to explain why surface lesions do not show a surface layer, why an outer layer later develops and what role fluoride plays as an inhibitor. The subsurface lesion leads to an increase of light scattering and altering the tooth's optical properties, which gives the WSL its characteristic white color. After 3-4 weeks this trend continues with an even greater subsurface mineral loss. If allowed to continue, the thin and porous enamel outer surface eventually collapses leading to cavitation.

4.3 PLAQUE pH AND FLUORIDE

Fluoride interferes with the de- and remineralization phases of the cariogenic process caused by plaque fermentation [26 and 27]. In addition, when fluoride is incorporated into dental tissue a more stable and less soluble mineral is produced [28]. It has been shown that at lower concentrations fluoride ions are absorbed onto the enamel crystals (creating fluorapatite), stabilizing their structure [29]. At higher concentrations calcium fluoride (CaF_2) or calcium fluoride-like material is formed and acts as a reservoir, releasing fluoride during cariogenic challenges [30]. CaF_2 is more stable and less soluble than fluorapatite [31]. The stability of CaF_2 depends on the amount of internal phosphate and the surrounding pH [32]

and 33]. This is because as the pH decreases, H^+ (protons) concentration increases; the phosphate surrounding the CaF_2 binds the protons and thereby freeing the CaF_2 . The CaF_2 thereby dissolves in the solution, freeing fluoride ions: $CaF_2 \leftrightarrow Ca^{2+} + F^-$.

Despite regular exposure of fluoride during treatment with fixed orthodontics, upper anterior teeth show a high prevalence of WSL development [34]. The reason for this is that a low saliva flow [35 and 36] to this area allows for a low pH to develop, inducing a rapid loss of free fluoride. Arneberg et al. (1997) showed a correlation between the plaque pH and plaque fluoride levels, finding the lowest plaque fluoride levels in the upper anterior teeth of patients undergoing fixed orthodontic treatment [37].

The lowest plaque pH achievable during a cariogenic challenge depends on three factors:

1. The dental plaque microbiota
2. The fermentable carbohydrate source
3. The diffusion rate of the carbohydrate and metabolites into and out of dental plaque

The minimum fermenting pH at the first central incisors was found to be about 4.5 [37]. It has been shown that decrease in plaque pH surrounding a WSL during a cariogenic challenge is even greater than for sound enamel for the first central incisors [38].

4.4 WHITE SPOT LESIONS (WSLs) AND THE ORTHODONTIC PATIENT

The most common iatrogenic effect of fixed orthodontic therapy is the development of WSLs [Fig. 1]. The bonding of brackets introduces new retention sites for dental plaque,

including sites on teeth such as the maxillary incisors, normally not susceptible to high levels of plaque accumulation. One study [34] found that 50% of individuals undergoing fixed orthodontic treatment had a WSL compared with 25% of controls. Another study found that 50% of the patients treated with fixed orthodontic appliances developed WSLs, compared to 11% in the control group [39]. Using quantitative light-induced fluorescence (QLF), an advanced technique allowing the detection of subclinical WSLs, Boersma et al. [40] found that 97% of orthodontically treated patients developed WSLs. Even 5 years after orthodontic treatment, patients had a significantly higher incidence of WSLs than a control group of patients who had not had orthodontic treatment [41].



Fig. 1. WSLs (indicated by white arrows) that have developed during orthodontic treatment due to suboptimal oral hygiene. Courtesy of Bjørn Øgaard.

The teeth with the highest prevalence of WSLs after treatment with fixed orthodontics are first molars, mandibular canines, premolars and upper lateral incisors [41].

It has been shown in previous cultural studies that the microbiota changes during fixed orthodontic treatment. Scheie et al. [42] found that after 3 months of treatment with fixed orthodontics *S. mutans* proportions in dental plaque surpassed pretreatment levels. Hallgren [43] found an increased proportion of both *S. mutans* and lactobacilli in dental plaque during treatment with fixed orthodontic appliances. In addition, recent molecular studies have associated *Actinomyces etunomyces*, *A. naeslundii* and *A. israelii* with WSLs [10 and 14].

The fate of WSLs can vary. After removal of the fixed orthodontic appliance they may partially fade due to remineralization [44 and 45] or toothbrush abrasion [46 and 47], or in extreme cases WSL cavitate and develop into frank caries. Esthetically WSLs may be a problem for the patient, especially the lesions occurring on the labial surface of the maxillary incisors. The problem can be quite severe in patients with very poor hygiene [Fig. 2].



Fig 2. Severe WSLs. Treatment had to be terminated due to poor oral hygiene. Courtesy of Bjørn Øgaard.

4.5 MOLECULAR IDENTIFICATION OF THE ORAL MICROBIOTA

While extremely useful, the identification and study of bacteria based on culture methods have their limitations. It is estimated that at least 50% of oral bacteria has not yet been cultured [14]. This means that studies of the oral microbiota based solely on these methods miss out a great deal of information regarding the bacteria present in the samples. In addition, bacterial identification based on culture cannot detect "viable but nonculturable" cells that are metabolically active but non-dividing [48].

The introduction of molecular identification techniques, also known as culture independent techniques, has allowed us to explore the microbiological world in more powerful ways. It has also had a profound impact on phylogenetic bacterial taxonomy (classification and identification of bacteria based on a hierarchy that shows their evolutionary relationships and bacterial nomenclature). Bacterial nomenclature is controlled by the 'International Code of Nomenclature of Bacteria'. The International Committee on Systematic Bacteriology (ICSB) maintains international rules for the naming of bacteria and taxonomic categories and for the ranking of them in the International Code of Nomenclature of Bacteria. Today's recommended approach to bacterial taxonomy is polyphasic (a multi-step process) [49], consisting of three methodologies:

1. Phenotypic: FAME fatty acid analysis
2. Genotypic: DNA-DNA hybridization, DNA profiling (ribotyping), MLST, GC ratio
3. Phenogenetic: individual genes analysis (16S rRNA), multi-gene, or whole genome analysis

Once identified, each bacterial species or subspecies is assigned to a taxonomic group consisting of: genus, family, order, class, phylum and domain. Furthermore, each species may consist of many different strains. Bacterial strains belong to the same species when there is a 70% or greater DNA-DNA hybridization and 98.5% or more of the 16S rRNA gene sequence.

4.5a 16S rDNA SEQUENCING

The noncoding 16S rRNA is also called SSU rRNA: small subunit ribosomal RNA. In the early 1970's Carl Woese from the University of Illinois pioneered the use of SSU rRNA for phylogenetic studies. His work established the presence of three domains of life; Bacteria, Archaea and Eukarya. Using SSU rRNA allows one not only to identify the bacteria, but by comparing the differences in their sequence one can study evolutionary differences between bacteria and hence construct phylogenetic trees. SSU rRNA is useful for molecular identification of bacteria because SSU rRNA is:

1. Universally distributed: found in all known bacteria
2. Functionally constant
3. Sufficiently conserved: it contains highly conserved regions and variable species specific regions
4. Of adequate length: Large enough (1,500 bases) to allow for species specific variation

All of these properties make 16S rRNA sequencing a powerful tool for bacterial identification.

While powerful, this method also has its limitations. Since the method does not rely on culture (live bacteria), it does not discriminate or differentiate between the 16S rRNA from

dead or living bacteria. Genetic material from dead bacteria can be incorporated into plaque and stay there for a long time. Another potential concern is that 16S rRNA undergoes limited change over time and that the variation present might not be adequate to provide good discrimination between closely related bacterial species. Other concerns arise from the steps needed for 16S rRNA isolation and amplification. DNA extraction must recover all DNA from both gram positive and gram negative bacteria. Gram positive bacteria have stronger cell walls making extraction more difficult. The method used must be powerful enough to extract DNA from even the toughest gram positive bacteria while at the same time avoiding DNA damage. Harsh DNA extractions may cause DNA shearing and lead to bias and artifacts like “chimeric” products [50]. Despite these limitations, 16S rDNA sequencing provides the basis of the newly revised version of Bergey’s Manual of Systemic Bacteriology.

Besides 16S rRNA, other phylogenetically informative genes may be used, e.g.,

1. 23S rRNA (LSU-rRNA): Longer, more informative but also more costly
2. Protein synthesis elongation factor Tu
3. Hsp60: Heat shock protein 60
4. Several tRNA synthases

4.5b POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction was developed by Kary Mullis in 1983 [51]. Only 10 years later he was awarded the Nobel Prize for this invention.

PCR selectively amplifies even a small amount of DNA several orders of magnitude, exponentially producing large amounts of the desired DNA sequence. Cycles of heating and cooling lead to DNA denaturation and enzymatic replication. After denaturation, primers (DNA oligonucleotides) attach to the ends of the DNA sequence of interest followed by replication with DNA polymerase (such as Taq polymerase).

Bias during the DNA amplification using PCR may occur [50 and 52]. The PCR primers may not amplify DNA from all bacteria with equal efficiency due to sequence differences, some might have 16S rDNA so different that the primers will not attach and amplify them at all. Oral samples also often contain blood. The heme acts as a PCR inhibitor by blocking the active site of DNA polymerase [53]. Other possible inhibitors for the PCR reaction include proteases, phenols and detergents. Bias of the PCR reaction may also occur due to the fact that different DNA sequences have different bond strengths and hence different denaturation levels.

4.5c PYROSEQUENCING

Pyrosequencing [54] is also called "sequencing by synthesis" because the method involves using a single strand of the DNA to be sequenced and then synthesizing its complementary strand. The method is based on detecting the activity of DNA polymerase using a luminescent enzyme (luciferase). A, C, G, and T nucleotides in solution are sequentially added and removed from the ssDNA to be sequenced. Light is produced when a nucleotide pairs with the template ssDNA.

Advantages of pyrosequencing compared to other methods are that biases in PCR amplification [55] are removed, speed and that no gels or nucleotide tags are needed [56]. A drawback compared to chain termination DNA sequencing methods is that the ssDNA lengths are shorter, making the reassembly more difficult. This can give a false picture of an inflated diversity [57].

4.5d THE HOMIM MICROARRAY

Microarrays are small solid-state silica plates etched using photolithography, which is also used to make computer chips. Once etched, oligonucleotide probes are bound on a membrane via polythymidine tails leaving them available for hybridization. These oligonucleotide probes are short (15-30 nucleotides) single stranded DNA sequences complementary to 16S rDNA target sequences. The HOMIM microarray stands for Human Microbial Identification Microarray and contains 16S rDNA probes for over 300 known oral bacterial species (including bacteria that cannot be cultivated). Using HOMIM, it is possible to perform a microbial community analysis on each plaque sample.

Microarrays are based on high throughput technology, and besides bacterial identification they may also be used for gene expression studies, for example in cancer. In that case the oligonucleotide 16S rDNA probes are replaced with probes for the genes in question.

Microarrays function similar to checkerboard hybridization [58]. The differences are that microarrays are a miniaturized version of the checkerboard, allowing for more samples to be analyzed simultaneously. Checkerboards use whole genomic probes for hybridization while microarrays use oligonucleotide probes. In addition, the detection of the results is

computer based: the light intensity of each cell in the microarray is analyzed by a computer. The HOMIM oligonucleotide probes are linked at their 5' end with fluorescent dyes. The probes that hybridize on the microarray radiate a fluorescent green color. The intensity of the color is proportional to the amount of hybridized 16S rDNA present [Fig. 3].



Fig. 3. Fluorescent dots indicate identified bacterial species. <http://mim.forsyth.org/homim.html>

Molecular technologies such as the HOMIM microarray can detect many more bacteria than traditional culture-based methods. However even HOMIM is far from being able to detect all bacteria present in dental plaque. On the bright side, being able to measure the composition and changes in a subset of the community, HOMIM still can tell us something about the dynamics of the overall bacterial community since there are interactions between the detectable subset and the other members of the community.

Microarrays have some limitations, including: crossover-hybridization that may cause false positives, and possible lack of hybridization causing false negatives [59]. A third possible problem is related to the probe length; a short probe is easier to make, but may not be sufficiently sensitive [60].

Besides microarrays, other methods of analyzing the 16S rDNA exist. Like microarrays, Fluorescent *in Situ* Hybridization (FISH) is based on hybridization. In addition to giving

information about the bacteria present, FISH can show us where in the plaque the identified bacteria are located. A drawback is that only a few bacteria can be identified per sample. Another related method is DGGE: Denaturing gradient gel electrophoresis, which can theoretically reveal single base DNA differences. DGGE separates PCR amplicons through a linear gradient of DNA denaturants: urea and formamide. The higher the GC content of the DNA, the harder it is to melt. A GC clamp can also be incorporated during PCR to give better resolution. When the fragments stop migrating they form individual fingerprints. 16S rDNA is most commonly used. A possible drawback with DGGE can be that it underestimates bacteria since bacteria in low numbers are not represented [61 and 62].

A similar method to DGGE is ribotyping. Ribotyping looks at bands, called DNA fingerprints. Digestion of the 16S rDNA is followed by gel electrophoresis and finally hybridization with rRNA gene probe. Drawbacks include that a database containing the “fingerprint” of all relevant bacteria is necessary and that up to 20 different restriction endonucleases are required.

4.6 CLINICAL CLASIFICATION OF WHITE SPOT LESIONS

Clinically visible WSL can be detected and scored by direct observation. Gorelick developed a system [Fig. 4] for doing this [34]. After drying the teeth with air, the WSLs were given the following scores:



Fig. 4. WSL grading system developed by Gorelick [34].

Recently new tools have emerged that enable the clinician to augment detection of enamel caries. Laser Fluorescence (LF) is one such tool. The device is commercially known as KaVo's (KaVo Dental, 11727 Fruehauf Drive, Charlotte NC 28273) DIAGNOdent, and uses a 655 nm diode laser [Fig. 5]. The laser can be shined on noncavitated, occlusal pit-and-fissure caries as well as smooth surface caries, allowing for early detection. The device measures laser fluorescence within the tooth structure. At 655 nm, a healthy tooth structure exhibits little or no fluorescence. However, a carious tooth structure will exhibit fluorescence, proportionate to the degree of caries, resulting in an increased reading on the DIAGNOdent display.

While useful in many clinical settings, this device does not suit the study of the size, shape and quantification of WSLs on buccal surfaces due to the fact that it can only examine the tooth surface point by point (the area being the same as the point of light emitted by the laser).



Fig. 5. DIAGNOdent device.

http://t0.gstatic.com/images?q=tbn:ANd9GcSgT7XicSmsHPCuhm9Uhl1HArQKT3Uh7xPzmopNRVU_KK3wCD9_

Another tool is the QLFTM method (Inspektor Research Systems BV, The Netherlands). The QLFTM system consists of hardware (a handpiece and a control box), a Windows PC and QLFTM software. This device is also based on the auto-fluorescence of teeth. Blue light (405 nm) is used to induce green fluorescence from within the dentine. A camera with a built-in long-pass filter is used to detect this green fluorescence. The filter on the camera does not allow the original blue light to enter the camera. This technique can be used to detect WSLs because they absorb and scatter the emitted green fluorescence, and this can be detected and quantified with the camera and PC software.

QLFTM has been used successfully to detect WSLs [63, 64 and 65]. However, this technique did not suit our needs since its size and weight of the equipment made it impractical to transport every time a patient was added to our study.

4.7 3-DIMENSIONAL ANALYSIS OF WHITE SPOT LESIONS

X-ray microtomography also called a micro computed tomography (CT), can be used to create a virtual 3-dimensional model of a WSL without destroying the original lesion [Fig. 6]. The method uses x-rays to create cross-sections of the WSL and then a computer program puts these sections together to produce a 3-dimensional virtual model. The micro-CT works in principal the same way as a clinical CT scanner does.

Creating a virtual 3-dimensional model allows the quantification of a WSL. This in turn makes it possible to compare different properties of WSLs including their surface area and depth.

X-ray microtomography has been used to evaluate the 3-D structure of both enamel and dentin caries with great success. Wong [66] used the technique to show for the first time the structure of dentin caries at the enamel-dentin junction. Taylor [67] showed that reliable algorithms can be created for accurate automatic quantification of natural occlusal caries lesions. In particular, WSLs have been evaluated with this technique with great success [68, 69 and 70].



Fig. 6. A μ CT device analyzing a sample in 3 dimensions.

<http://www.microphotonics.com/images/skyscan1172.gif>

4.8 WHITE SPOT LESION COLOR QUANTIFICATION

The perceived color of a tooth and its WSL varies depending on the light setting the tooth is exposed to. In addition, while the human eye can easily detect that a WSL is indeed “white”, it is more difficult to assign a value to this “white” color and quantify it. By taking pictures of WSLs with the same camera, under the same conditions and under the same controlled lighting conditions it is possible to determine the properties of the WSL’s color (thereby quantifying it) in terms of its RGB (Red, Green, Blue) coordinates [Fig. 7]. This then makes it possible to compare different WSLs with each other.

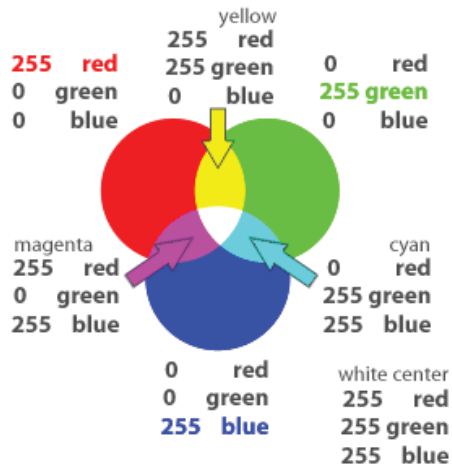


Fig. 7. A color coordinate system. <http://ephotospros.com/Portals/0/rgb-theory.png>

Many commercial color detection devices use these principles, including X-Rite

ShadeVision™ [Fig.8] and Shade-X™.



Fig. 8. X-Rite ShadeVision™ http://www.xrite.com/images/widget/Markets_Dental2.jpg

5. AIMS OF THE STUDY

Thousands of articles have been written about the microbiology of the dental caries lesion.

However, most of these studies were done by culture which can recover only about 50% of

the bacteria present. In addition, most of the articles have focused on only a few bacteria

(usually *S. mutans* and lactobacilli). Research in the field of the microbial etiology of caries has been and is limited by the technological means we have at our disposal to study the microbiota. The recent advances in biotechnology have allowed us to move away from traditional culture-based methods of studying the microbiota with high-throughput genetic methods.

Technological advances have also led to new tools that allow 3-dimensional properties of WSLs to be studied and for the quantification of their clinical color.

To gain a more complete understanding of the nature of WSLs the following studies were done:

- 1)** Using an *in vivo* model in a longitudinal cohort study to investigate shifts in the microbial community composition associated with the development of enamel caries. **(Paper I)**
- 2)** Gain a more complete understanding of how fixed orthodontic appliances change the supragingival microbial profile during the first 5 months of treatment. **(Paper II)**
- 3)** Calculating the volume of white spot lesions (WSLs) using micro computed tomography (μ CT) and to determine which clinical attribute of the WSL could better predict its volume: the clinically visible WSL surface area or its color intensity. **(Paper III)**

6. SUMMARY OF RESULTS

6.1 PAPER I: Microbial community succession on developing lesions on human enamel

White spot lesions were generated *in vivo* on human teeth predetermined to be extracted for orthodontic reasons. The bacterial microbiota on sound enamel and on developing carious lesions were identified using the Human Oral Microbe Identification Microarray (HOMIM), which permits the detection of about 300 of the approximate 600 predominant bacterial species in the oral cavity.

After only 7 weeks, 75% of the targeted teeth developed clinically diagnosed white spot lesions (8 individuals, 16 teeth). The microbial community composition of the plaque over white spot lesions differed significantly as compared to sound enamel. Twenty-five bacterial taxa, including *S. mutans*, *Atopobium parvulum*, *Dialister invisus*, and species of *Prevotella* and *Scardovia*, were significantly associated with initial enamel lesions. In contrast, 14 bacterial taxa, including species of *Fusobacterium*, *Campylobacter*, *Kingella*, and *Capnocytophaga*, were significantly associated with sound enamel.

Of note, 25% of the teeth did not develop a lesion after 7 weeks. Consequently, we compared the changes in microbiota on pairs of samples between baseline and for those samples that did not develop a lesion. For this small sample size (n=23 premolars), there were no taxa that were significantly more prevalent at baseline or at the final visit.

Communities with Plaque Index 2 were significantly different from those with plaque index 0 and 1. The average number of taxa for Plaque Index 0, 1 and 2 was 38.50, 44.13 and 53.68

respectively. The Wilcoxon Signed-Rank Test was used for each pair; plaque index 0 and 2 were compared: p-value=0.0014, and plaque index 1 and 2: p-value= 0.0247.

There were differences in the prevalence of specific taxa in supragingival plaque taken from WSL 1 sites as compared to supragingival plaque from WSL 3 sites at the final visit. Four taxa, namely *Gemella morbillorum*, *Selenomonas* sp. OT136/OT148, *Megasphaera micronuciformis*, and *S. sputigena*, were more prevalent in WSL 1 samples (no lesions), (p-value < 0.01). However, the results were not significant (e.g. p-value > 0.05) when adjusted for multiple comparisons.

6.2 PAPER II: Changes in the supragingival microbiota surrounding brackets of upper central incisors during orthodontic treatment

The aim of this study was to determine how fixed orthodontic appliances affect the microbiota of supragingival plaque over 5 months.

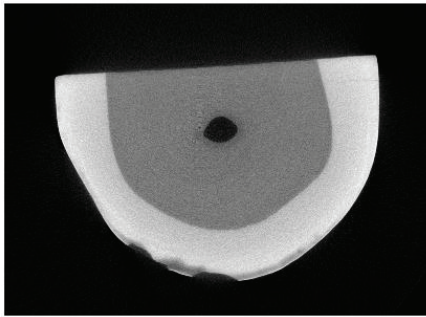
Twenty individuals of Scandinavian origin, ages 10 - 16 years, were included. All subjects were fitted with fixed orthodontic appliances in both the maxillary and mandibular tooth arches. Pooled supragingival plaque samples from the labial surface of the two maxillary central incisors were collected before bonding (T1), and afterwards at 4 weeks (T2), 3 months (T3) and 5 months (T4). The plaque index (PI) was recorded at each sampling. The gingival status was documented at T1 and T4 by using standardized clinical photographs. The plaque microbiota was identified using the Human Oral Microbe Identification Microarray (HOMIM).

Increased plaque levels were recorded after bonding, however the increase was not significant. The prevalence of gingivitis at the maxillary central incisors increased from 25% at T1 to 74% at T4. No significant changes of the plaque microbiota from the sample area were detected during the 5-month period.

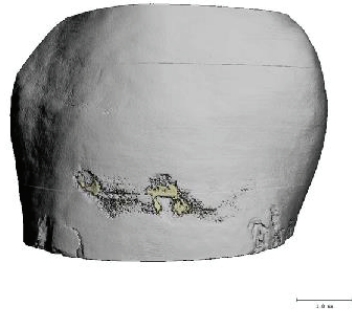
Although trends toward a microbiota containing more periodontitis- and caries-associated bacteria were detected, the changes were not severe enough to be significant. Treatment with fixed orthodontics does not necessarily shift the microbiota to a more pathogenic composition.

6.3 PAPER III: Clinical color intensity of white spot lesions (WSLs) may be a better predictor of enamel demineralization depth than traditional WSL clinical grading

WSLs were induced in 8 patients *in vivo* on 23 healthy premolars destined for extraction due to orthodontic treatment, using specially designed plaque retaining orthodontic bands. After 7 weeks the premolars were extracted. Following extraction, the resulting WSLs were photographed and clinically graded. The teeth were analyzed by μ CT [Fig. 9].



μCT cross-sectional image showing the WSL



μCT 3D reconstruction of tooth and WSL

Fig. 9. A WSL detected by μCT. Bar = 1mm

After 7 weeks, 70% of the teeth had developed clinical WSLs. Clinically the size of the WSLs varied from minor to severe. The μCT data showed that the gray scale value (which is proportional to mineral density) of healthy enamel did not vary between the teeth. However, in all cases the WSL gray scale values were lower than the healthy enamel values. The volumes of the WSLs varied from 0 mm³ to 1.2931 mm³. Fig. 9 shows a representative cross-sectional μCT image of one of the teeth analyzed with the μCT and illustrates how the μCT software can compile the cross-sectional images to produce a 3D image of the WSL. A significant correlation (gamma coefficient = 0.816 with power = 0.999) between WSL clinical score and the WSL mean color differences value was found. The WSL clinical score correlated somewhat poorly (gamma coefficient = 0.514 with power = 0.721) with the WSL volume. A significant correlation (Spearman's coefficient = 0.681 with power = 0.962) was found between the WSL mean color difference and the WSL volume.

Not surprisingly, our study found that the WSL clinical score correlated strongly with the WSL mean color difference, meaning that WSLs covering a larger tooth surface are also often whiter than smaller surface lesions. More interestingly we found a weak correlation between WSL volume and clinical WSL score, meaning that the traditional WSL score is a rather weak indicator of enamel demineralization depth. Our results show that there is a significant correlation between WSL intensity and lesion volume.

7. GENERAL DISCUSSION

7.1 MICROBIOTA OF WHITE SPOT LESIONS

WSL development on labial enamel surfaces in orthodontic patients with suboptimal oral hygiene is by far the most common iatrogenic effect of fixed orthodontic therapy.

Orthodontic patients used a considerable amount of time and money to align their teeth and therefore expect and hope for a good esthetic result. Multiple WSLs visible after debonding are a rather disappointing adverse effect of orthodontic treatment.

Orthodontists (by giving oral hygiene instruction) and companies developing orthodontic equipment (by developing appliances with low plaque retention) both work at reducing the likelihood of WSL development, however we are still far from a perfect solution.

As stated by the 'ecological plaque hypothesis', it is the shift in the microbial composition that is responsible for oral diseases. Papers I and II have illustrated this fact. In Paper I significant shifts in the microbial composition also led to clinically visible WSLs while in Paper II, the microbial compositions shifts were minor due to satisfactory oral hygiene and no WSLs developed.

In Paper I, the bacterial diversity of the predominant oral microbiota associated with development of cariogenic lesions was determined using HOMIM in an *in vivo* model. Our results indicate that the microbiota on intact enamel was significantly different from that of the microbiota associated with white spot lesions developed for 7 weeks under protected metal bands. The microbial communities in dental plaque associated with caries included

species of the genera *Acidaminococcaceae*, *Streptococcus*, *Lactobacillus*, *Veillonella*, *Prevotella*, *Solobacterium*, *Scardovia*, and *Atopobium*. Some of these were associated with the increasing severity of WSLs such as *S. wiggsiae*, *S. salivarius* and *V. atypica* and might play important roles in the process of caries development. The *in vivo* model of microbial community succession provided novel insights into the microbial community shifts associated with the development of WSLs, and likely dental caries. Consequently, this model can be used to study the efficacy of diet, antibiotics, and other prophylactic and restorative treatments, and may help design more effective interventions and preventions.

Paper II investigated the microbial community shift surrounding orthodontic brackets during the first 5 months of treatment. Our hypothesis was that the microbial community on enamel susceptible to WSL development changes significantly during orthodontic treatment. In our subject population however, plaque levels were not significantly increased after placement of brackets and there were also no significant shifts in the microbial community. Our hypothesis that fixed orthodontic appliances increased plaque levels and resulted in a shift of the microflora had to be rejected. This came as a surprise considering that many papers have reported significant microbial shifts [42]. This may be due to different sampling sites; upper incisors are easily accessible for oral hygiene. Another reason may be that the subjects were extra meticulous in their oral hygiene habits because they were participating in the study. Our results may also have been affected by the fact that our patients wore both metal and elastic ligatures. It has been shown that elastic ligatures are more prone to plaque retention [71]. However even patients with metal ligatures can develop WSLs, meaning that both types of ligatures have the potential to alter the microbial community. One may speculate that if only elastic ligatures were worn, a slightly more meticulous oral

hygiene would have been required in order to achieve the same level of plaque removal and therefore could have altered the microbial community to a greater extent.

WSL development on upper incisors is common [34], yet we found only minor shifts in the microbial community in this area. We hypothesize that this may be explained by the fact that it has been shown that plaque on the buccal surfaces of upper incisors has lower resting pH than plaque on other sites [37] due to low levels of saliva flow in this area [35 and 36]. This might be especially true for patients with overbite and incompetent lips. This may therefore mean that a less than 'normal' cariogenic (acidogenic) microbial composition can produce adequate levels of acid to demineralize the enamel and create WSLs.

The results show that placement of orthodontic appliances does not in itself lead to disturbances of the microbial composition. Instead, the microbial composition depends on plaque levels and oral hygiene. These results fit with what we observe clinically; that orthodontic patients with satisfactory oral hygiene are at low risk of developing WSLs. However, one important point to remember is that G.V. Black showed that just 2 weeks of plaque stagnation can lead to clinically visible WSLs. This may explain why Hadler et al. [39] detected a minor increase in WSLs even in patients with good oral hygiene (i.e. some of the patients may have had poor hygiene between visits and this might not have been detected if the hygiene improved again prior to the next visit). This same explanation may partly explain why Paper II found (surprisingly) no significant changes of the microbial community.

In recent years the scientific community has started to move away from blaming oral diseases on single bacterial species. This has complicated things greatly. 16S rRNA

sequences have given us new knowledge and understanding of the caries process, however we are starting to understand that studying the oral microbiome is even more complex than expected. The oral microbiome contains DNA from bacteria, archaea, fungi, viruses and protozoa, some of which may be dead.

Studies in the future will have to take the concept of phenotypic plasticity and epigenetics into account. Even if a future version of the HOMIM microarray containing all possible 16S rRNA probes will give us a complete picture of what the microbial composition looks like, the phenotypes of the bacteria can still be quite different depending on the environment in which the organisms are growing [72]. This means that the same bacteria can be cariogenic or non cariogenic depending on growing conditions *in vivo*. It has been shown that pH, oxygen and redox conditions, carbohydrate source and availability, and population density have a profound impact on the gene expression profiles in oral streptococci [73]. Secondly, individual species of oral bacteria display a large degree of genetic heterogeneity [74]. Different clones of the same species may display different properties. To address this problem we must first know which eventual clones are pathogenic and then develop specific 16S rRNA probes to target them. This is a demanding task due to the huge numbers of clones and since many oral bacteria are difficult to grow in culture. Clearly, more emphasis should be placed on culturing of not yet cultured bacteria.

In addition, simply finding which species are actually present in plaque is still a challenge. Different molecular techniques give different results. For example, a study using pyrosequencing in 2012 identified 186 novel taxonomic units in human dental plaque previously undetected by PCR [75]. In another sequencing study [76] 1,372 OTUs were

detected, of which 205 were named species while the about 800 remaining OTUs represented very low abundance phenotypes.

The results of different techniques are being gathered and discussed to try to make sense of the composition of the oral microbiome [77]. They have shown that the oral cavity is only second to the colon in terms of species richness. Interestingly, the aim of The Human Microbiome Project [78] is to determine the microbiome of different sites on the body.

The ultimate aim of research of the type done in Papers I and II is to gain a more fundamental understanding of the microbiota involved in the caries process hoping that this knowledge may help us eradicate dental caries - one of the most common diseases on the planet. Today there are many examples of how research in the field of oral microbiology has affected the treatment of caries clinically; from bacterial saliva tests to antimicrobial agents in toothpastes and dental materials. Sometimes research also leads to removal of antimicrobial agents from toothpastes as was the case with triclosan, which turned out to create resistance in bacteria. As Papers I and II have shown, the composition of plaque on healthy enamel surfaces and caries lesions is a very complex biofilm consisting of many species. Therefore focusing on inhibiting/eradicating single species will probably not be fruitful. Exciting new research has the goal of inhibiting the oral biofilm development (and therefore targets a broad spectrum of oral bacteria, without causing resistance) by using quorum sensing-disrupting thiophenones [79]. If found to be clinically effective and safe this research may give clinicians another tool for caries prevention.

7.2 3-DIMENSIONAL AND COLOR PROPERTIES OF WHITE SPOT LESIONS

Building on previous research and by using modern technologies it was possible to study WSLs in new ways. By using the μ CT to study WSLs in three dimensions and computer software in conjunction with a digital camera we showed that simply noting the color intensity of WSLs could be used by dentists as a simple chair side diagnostic method to predict the depth of the lesion.

Physical properties of WLSs were studied in this thesis in order to discover how the color of WSLs related to how deep the lesion has penetrated into the tooth surface. This is because the depth will influence the prognosis, which is of interest to both the patient and the dentist. With commonly available tools in the dental office, there is no way to be certain of how deep buccal WSLs have penetrated into the tooth surface *in situ* short of drilling into them.

Not surprisingly, our study found that the WSL clinical score correlated strongly with the WSL mean color difference, meaning that WSLs covering a larger tooth surface are also often whiter than smaller surface lesions. More interestingly we found a weak correlation between WSL volume and clinical WSL score, meaning that the traditional WSL score is a rather weak indicator of enamel demineralization depth. This might seem surprising; however the clinical WSL score is based mostly on the WSL surface area. Even though a large part of a buccal surface has developed a WSL, this does not automatically mean that the lesion has penetrated deep into the enamel. Our results show that there is a significant correlation between WSL intensity and lesion volume. The human eye can detect about 10 million colors. The eye is one of the most powerful tools a dentist possesses and our μ CT

results indicate that a simple visual inspection of a WSL color can predict (to some degree) its level of penetration into the enamel.

It has been shown that a correlation exists between the WSL color and the level of penetration. This may aid the dentist in predicting the prognosis. However the correlation between the WSL color and the level of penetration was not very strong, meaning that only looking at the color of the WSL is not enough to predict the level of penetration with certainty. An interesting question to ask is; why this is so? Part of the answer may be that since the enamel over WSLs is porous, the WSLs can take up dyes and stain the lesion hence reducing the intensity of the white color. This discoloration may be due to many different sources: Maillard pigments (a form of nonenzymatic browning), melanins, and lipofuscins, and the uptake of food dyes, metals, and bacterial pigments [80]. Another reason can be due to the fact that mechanical removal of plaque also leads to abrasion of the rough surface enamel. This leaves a smoother surface lesion with reduced light scattering properties and therefore a slightly different color of white [81 and 82]. Using the same reasoning we can come to the conclusion that an active WSL and an arrested WSL of the same depth could be different colors of white since active enamel caries lesions are known to have more porous surfaces. Yet other factors complicating the relationship between WSL color and lesion depth is the fact that the buccal surfaces of teeth are curved, therefore the light reaches the WSLs at different angles depending on where they are located and hence slightly influencing the WSL color properties. In addition, it has been shown that the optical properties of the tooth surface are affected by variations in enamel characteristics such as mineral density, crystal size, and prism orientation [83]. Finally, we must consider that our correlation between WSL color and WSL depth could have been even stronger if we had

used more advanced equipment for the color detection. A paper published in 2013 studied the color properties of WSLs using an advanced spectroradiometer to determine the CIE $L^*a^*b^*$ color coordinates (which are closely related to the human visual response) of WSLs [84]. This paper found that WSLs lead to an increase in lightness (L^*) and a decrease in yellowness (b^*) of the tooth surface.

Another factor to consider is the difference between the μ CT image and the physical WSL. Computer algorithms interpret and processes the data collected by the x-ray detector and reconstitute an image of the scanned WSL. During this step the computer is creating many 2-dimensional “slices” of the 3-dimensional WSL. In addition, the algorithms are removing artifacts created by unwanted x-ray scattering and other image distortions. While this method can create a reliable representation of the actual WSL, the image we see will differ depending on what algorithms we choose and therefore will be a source of error. Another important source of error is due to the fact that sound enamel and enamel containing a WSL have a similar threshold density (especially shallow lesions), meaning that it can be difficult to differentiate between the two on the μ CT image. This is solved by a method of image segmentation called ‘thresholding’, where the operator finds an appropriate cutoff value that allows the computer to differentiate between sound enamel and a WSL. Some information is ultimately lost during this step and it is important to realize this part of the image processing is subjective and will vary between operators.

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Microbial community succession on developing lesions on human enamel

Lino Torlakovic^{1,2}, Vanja Klepac-Ceraj³, Bjørn Øgaard², Sean L. Cotton³, Bruce J. Paster^{3,4} and Ingar Olsen^{1*}

¹Department of Oral Biology, University of Oslo, Oslo, Norway; ²Faculty of Dentistry, Institute of Clinical Dentistry, University of Oslo, Oslo, Norway; ³Department of Molecular Genetics, The Forsyth Institute Cambridge, MA, USA; ⁴Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Boston, MA, USA

Background: Dental caries is one of the most common diseases in the world. However, our understanding of how the microbial community composition changes *in vivo* as caries develops is lacking.

Objective: An *in vivo* model was used in a longitudinal cohort study to investigate shifts in the microbial community composition associated with the development of enamel caries.

Design: White spot lesions were generated *in vivo* on human teeth predetermined to be extracted for orthodontic reasons. The bacterial microbiota on sound enamel and on developing carious lesions were identified using the Human Oral Microbe Identification Microarray (HOMIM), which permits the detection of about 300 of the approximate 600 predominant bacterial species in the oral cavity.

Results: After only seven weeks, 75% of targeted teeth developed white spot lesions (8 individuals, 16 teeth). The microbial community composition of the plaque over white spot lesions differed significantly as compared to sound enamel. Twenty-five bacterial taxa, including *Streptococcus mutans*, *Atopobium parvulum*, *Dialister invisus*, and species of *Prevotella* and *Scardovia*, were significantly associated with initial enamel lesions. In contrast, 14 bacterial taxa, including species of *Fusobacterium*, *Campylobacter*, *Kingella*, and *Capnocytophaga*, were significantly associated with sound enamel.

Conclusions: The bacterial community composition associated with the progression of enamel lesions is specific and much more complex than previously believed. This investigation represents one of the first longitudinally-derived studies for caries progression and supports microbial data from previous cross-sectional studies on the development of the disease. Thus, the *in vivo* experiments of generating lesions on teeth destined for extraction in conjunction with HOMIM analyses represent a valid model to study succession of supragingival microbial communities associated with caries development and to study efficacy of prophylactic and restorative treatments.

Keywords: white spot lesions; caries; HOMIM; molecular microbiology

Received: 19 December 2011; Revised: 7 February 2012; Accepted: 22 February 2012; Published: 14 March 2012

Enamel caries, one of the most common human diseases, is considered a multi-factorial disease involving interactions among the oral microbiota, host immune system, diet, saliva and properties of the tooth surface. The ecological plaque hypothesis (and its many variations) (1–5) stressed the importance of changes in local environmental conditions as causes for shifts in the proportions of species within a mixed plaque population. The ecological plaque hypothesis also states that potentially cariogenic bacteria may be present in clinically low levels in dental health. The implications are that caries could be controlled not only by targeting cariogenic bacteria but also by interfering with local

factors responsible for driving the harmful shifts in the microbiota. Culture-independent methods based on molecular analysis of 16S rRNA genes have led to an expanded list of caries-associated species in addition to *Streptococcus mutans*, including species of *Actinomyces*, *Abiotrophia*, *Atopobium*, *Bifidobacterium*, *Lactobacillus*, *Olsenella*, *Pseudoramibacter*, *Scardovia*, *Selenomonas* and *Veillonella* (6–11). In particular, recent molecular studies (10) have associated *Actinomyces gerencseriae*, *A. naeslundii* and *A. israelii* with initial enamel lesions also known as white spot lesions (WSLs). Furthermore, many studies support the view that caries can develop in the absence of *S. mutans* (5, 10, 12). Consequently, several

bacterial species, either alone or as a group, other than *S. mutans* may also play major roles in caries development (1, 5, 13, 14).

In this study, WSLs were artificially induced *in situ* on the buccal surface of premolars intended for extraction due to orthodontic treatment. The microbial communities were followed longitudinally in each subject for seven weeks and the communities of sound enamel were compared with those of WSLs by using the Human Oral Microbe Identification Microarray (HOMIM), which is a high-throughput method capable of simultaneously detecting about 300 oral predominant bacterial species including those that have not yet been cultivated (11, 15) (<http://mim.forsyth.org>).

Material and methods

Subject population

The subject population consisted of five male and three female individuals of Scandinavian origin, chosen from an orthodontic clinic for the study. All were 10 to 14 years old and had premolars scheduled for extraction due to orthodontic reasons. Only subjects with little to no past caries experience were included in the study. A total of 23 premolars were used and the premolars had no signs of clinical WSLs at baseline. For all scheduled study visits, participants did not brush their teeth the morning prior to sampling and had no breakfast within an hour before sampling. All appointments were scheduled between 9 am and 12 pm. While enrolled in the study, participants brushed their teeth with the non-fluoride toothpaste Solidox® (Lilleborg AS, Oslo, Norway) and did not use

any fluoride or antimicrobials, such as chlorhexidine or triclosane. The study was approved by the Regional Ethics Committee (REK sør-øst, PB 1130, Blindern, Oslo, Norway). The subjects and their guardians signed an informed consent form before the start of the study.

Clinical procedures

Orthodontic bands with two metal posts (0.8 mm thick) welded onto the inner buccal surface specifically designed for plaque accumulation (16), were banded on premolars destined for extraction during the first visit (Fig. 1). The buccal surface of the premolars was visually inspected before the banding. No signs of previous or current caries activity were detected. Temp Bond NE (Kerr Corporation, Orange, CA, USA) was used to cement the bands in place (17). Any residual cement between the buccal tooth surface and the band was removed. Plaque samples were taken from premolars at baseline (immediately before banding) and at the final visit 7 weeks after banding.

Care was taken not to touch the premolar area prior to plaque sampling. A lip spreader was used to isolate the premolars and the buccal surfaces were allowed to air dry before sample collection. The same examiner collected all samples with a sterile orthodontic wire (Fig. 1) and immediately suspended each sample in 300 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The samples were transported to the laboratory using the Nalgene™ Labtop Cooler (−20°C), before placing them at −80°C until further processing. For baseline sampling, the buccal enamel surface where the band would be placed was sampled with a probe. At the final visit, the probe was inserted into the space between the band and the



Fig. 1. Orthodontic bands designed for plaque retention. The bands were placed *in vivo* on premolars.

buccal tooth surface and the plaque sample was collected from the enamel surface.

After collecting the sample at the final visit, the examiner extracted the premolars and the orthodontic treatment was continued as planned by the orthodontist. The buccal surface of the extracted premolars was carefully cleaned with water, air dried and then the buccal lesion was given a score in the following manner (Fig. 2): no WSLs = 1, minor WSLs = 2 and severe WSLs = 3 (18).

DNA isolation and HOMIM protocol

DNA was isolated from clinical samples using a Ready-Lyse™ Lysozyme Solution (Epicentre) for overnight incubation before using a MasterPure DNA Purification Kit (Epicentre) according to the manufacturer's directions. Purified DNA samples were analyzed using HOMIM, which was developed to simultaneously detect ~300 of the most prevalent oral bacterial species. The method is described in detail elsewhere (15). Briefly, 16S rRNA-based, reverse-capture oligonucleotide probes (typically 18–20 bases) were printed on aldehyde-coated glass slides. 16S rRNA genes were PCR amplified from DNA extracts using 16S rRNA universal forward and reverse primers and labeled via incorporation of Cy3-dCTP in a second nested PCR. The labeled 16S rRNA gene amplicons were hybridized overnight with probes on the slides. After washing, the microarray slides were scanned using an Axon 4000B scanner and crude data were extracted using GenePix Pro software.

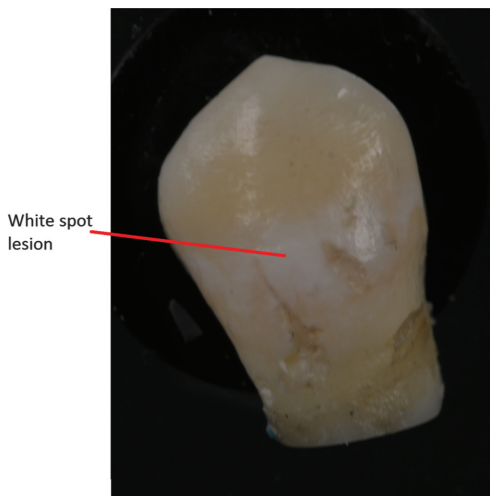


Fig. 2. Resulting white spot lesion after 7 weeks of plaque retention. The photograph was taken after the removal of an orthodontic band.

Analysis of microbial community profiles

Microbial community profiles were generated from image files of scanned HOMIM arrays using a HOMIM online analysis tool (available at: <http://bioinformatics.forsyth.org/homim/>). The detection of a particular taxon in a sample was determined by the presence of a fluorescent spot for that unique probe. A mean intensity for each taxon was calculated from the hybridization spots of the same probe and the signals were normalized and calculated as previously described (15). The range of signal levels was obtained by raising normalized signal intensities to the power of 0.3. Any original signal that was less than two times the background value was reset to 1 and was assigned to the signal level 0; indicating absence of a particular taxon in a sample. The remaining signals, those greater than 1, were categorized into scores from 1 to 5; corresponding to different signal levels. To determine how bacterial community composition varied across samples, we compared total hybridization profiles obtained by HOMIM arrays for each sample using correspondence analysis (CoA) in MeV 4.6 (19). Analysis was performed on the absolute intensity of HOMIM data (frequency of scores from 0 to 5) as well as binary data (presence/absence). The prevalence of each taxon was computed for each subject and averaged within groups. Wilcoxon Rank Sum test and *t*-test were used to identify statistically significant differences between groups (baseline and final visit; WSLs 1, 2 and 3; and plaque indices 0, 1 and 2). A *p*-value of <0.05 was considered significant. False discovery rate (FDR) using Benjamini-Hochberg correction was used to control for multiple hypothesis. For the cluster analysis, Pearson correlation was used as a distance metric selection. JMP 9.0 (www.jmp.com/) and R Statistical Package (www.r-project.org/) were used in statistical analyses.

Results

Succession of microbial communities

At the end of seven weeks at the final visit, 75% of the premolars had developed WSLs on their buccal surfaces underneath the retention band (Fig. 2). There was a clear shift of the microbiota from plaque found on sound enamel (baseline) as compared to plaque retained after seven weeks (final visit). Microbial communities at baseline were found to be significantly different ($p < 0.001$; *t* test) from the microbial communities at the final visit (Figs. 3 and 4). For each visit, samples had a bacterial 'signature' for each individual; samples from the same individual, in general, were more similar to one another than when taken from two different individuals (Fig. 3).

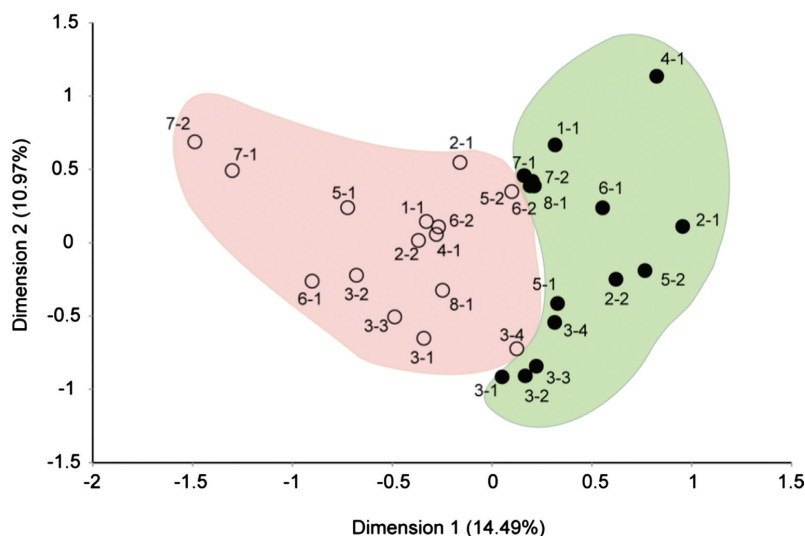


Fig. 3. Plot of correspondence analysis (CA) of the enamel bacterial community using binary data (presence/absence). Each circle represents one community from a single visit from an individual patient. Only communities with data for both the baseline and final visit are plotted and labeled by color according to visit. The baseline samples are labeled with solid circles; samples from the final visit (7 weeks later) are labeled with open circles. Numbers next to each circle indicate a patient and tooth number. The percentages of variation described by the correspondence analysis coordinates are shown in parentheses. Microbial communities from the baseline visit are significantly different ($p < 0.001$; t test) from the final visit.

Microbial community of dental health (baseline)

Fourteen bacterial taxa were found to be prevalent in plaque covering sound enamel at baseline ($p < 0.01$; Fig. 4). The most commonly detected species in greater than 80% of samples were *Gemella morbillorum*, *Kingella oralis* and *Capnocytophaga granulosa*. All species have been detected previously in supragingival and subgingival plaque (15). All but *Campylobacter concisus* and *Campylobacter showae* are capable of fermenting carbohydrates to acid, although *C. concisus* and *C. showae* can produce acetate and/or succinate with formate and fumarate in the culture medium (20).

Microbial community of the initial caries lesion developed after 7 weeks (final visit)

Twenty-five bacterial species were significantly increased in plaque behind the retaining bands after seven weeks (Fig. 4). Many of these species have been previously associated with caries development, such as *Streptococcus mutans*, other species of *Streptococcus* and species of *Lactobacillus*. Other species less known to be associated with caries development included *Atopobium parvulum*, *Dialister invisus*, and species of *Prevotella* and *Scardovia*.

Of note, 25% of teeth did not develop a lesion after seven weeks. Consequently, we compared the changes in microbiota on pairs of samples between baseline and for those samples that did not develop a lesion. For this small

sample size, there were no taxa that were significantly more prevalent at baseline or at the final visit.

Microbial community with respect to plaque index

Communities with Plaque Index 2 are significantly different from plaque indices 0 and 1. The average number of taxa for Plaque Index 0, 1 and 2 was 38.50, 44.13 and 53.68, respectively. The Wilcoxon Signed-Rank Test was used for each pair; plaque indices 0 and 2 were compared: p -value = 0.0014, and plaque indices 1 and 2: p -value = 0.0247.

Microbial community of initial caries lesions with respect to severity of disease

There were differences in the prevalence of specific taxa in supragingival plaque taken from WSL 1 sites as compared to supragingival plaque from WSL 3 sites at the final visit. Four taxa, namely *Gemella morbillorum*, *Selenomonas* sp. OT136/OT148, *Megasphaera micronuciformis*, and *Selenomonas sputigena*, were more prevalent in WSL 1 samples (no lesions), ($p < 0.01$). However, due to the low number of samples (n), the results were not significant (e.g. $p > 0.05$) when adjusted for multiple comparisons.

Discussion

The results clearly showed an ecological shift in dental plaque over developing WSLs in enamel. Many bacterial species, including typical caries-associated species such as

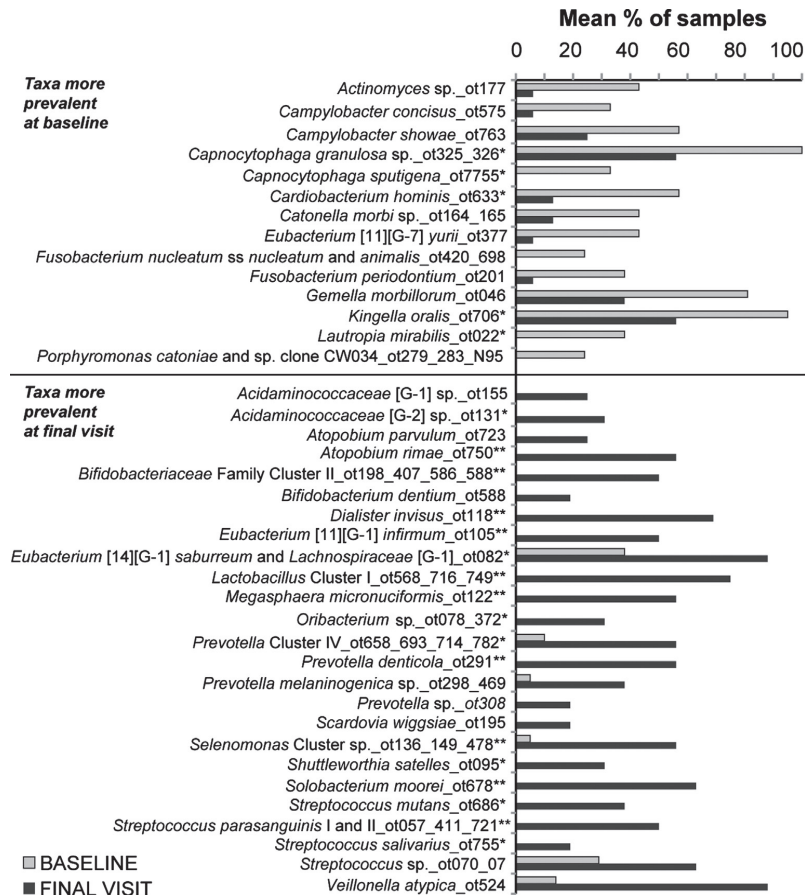


Fig. 4. Mean frequencies of bacterial probes that were significantly different ($p < 0.05$; Wilcoxon test) among subjects at baseline and final visit. *Indicates adjusted p (Benjamini/Hochberg) values $p < 0.01$ and ** $p < 0.001$.

S. mutans and *Lactobacillus* spp., increased with the development of WSLs. Consequently, the bacterial community composition associated with the progression of WSL is specific and much more complex than previously believed and should be explored in future studies. Our results not only confirmed previous studies (6, 8, 10, 11), but additional species or phylotypes that were significantly associated with the longitudinal progression of caries in the secondary dentition were identified (Table 1) (10).

Many of the bacterial species found to be increased in plaque covering sound enamel and in plaque behind the plaque retaining bands are capable of producing acid. Bacteria capable of producing caries need to be both acidogenic (able to produce acid) and aciduric (able to survive in an acid environment) (21). It is noteworthy that

non-mutans streptococci, such as those found in WSLs, are capable of acidogenesis at low pH and typically outnumber mutans streptococci. Consequently, several species of non-mutans *Streptococcus* have been implicated in caries production (13). Indeed, in this study several species or phylotypes were significantly more prevalent in WSLs (Fig. 4).

Our results provided strong evidence in support of the ecological plaque hypothesis, that is, enamel caries develops due to changes in local environmental conditions (plaque retaining bands), which disrupt the natural balance between plaque and the host, leading to enrichment of organisms that can potentially cause caries. In addition, our results showed that many bacterial species other than *S. mutans* and lactobacilli are associated with caries *in vivo*.

Table 1. Comparison of statistically significant bacterial taxa associated with carious lesions in the secondary dentition

Species/phylotype	This study	Aas et al. (10)	
	WSL	WSL	caries
<i>Acidaminococcaceae</i> sp. OT131	+		
<i>Acidaminococcaceae</i> sp. OT155	+		
<i>Atopobium</i> spp.	+		+
<i>Bifidobacterium</i> spp.	+		
<i>Campylobacter gracilis</i>		+	
<i>Dialister invisus</i>	+		
<i>Eubacterium infirmum</i>	+		
<i>Eubacterium</i> sp. OT082	+		
<i>Lactobacillus</i> spp.	+		+
<i>Leptotrichia</i> sp. GT018			+
<i>Megasphaera micronuciformis</i>	+		
<i>Oribacterium</i> sp. OT078	+		
<i>Prevotella denticola</i>	+		+
<i>Prevotella melaninogenica</i>	+		
<i>Prevotella</i> sp. OT308	+		
<i>Propionibacterium</i> sp. FMA5			+
<i>Scardovia wiggsiae</i>	+		
<i>Selenomonas</i> sp. OT136	+	+	
<i>Shuttleworthia satelles</i>	+		
<i>Solobacterium moorei</i>	+		
<i>Streptococcus parasanguinis</i>	+		
<i>Streptococcus</i> sp. OT070	+		
<i>Streptococcus salivarius</i>	+	+	+
<i>Streptococcus mutans</i>	+		+
<i>Veillonella</i> spp.	+	+	+

In summary, the bacterial diversity of the predominant oral microbiota associated with development of cariogenic lesions was determined using HOMIM in an *in vivo* model. Our results indicate that the microbiota on intact enamel was significantly different from that of the microbiota associated with WSLs developed for seven weeks under protected metal bands. The microbial communities in dental plaque associated with caries included species of the genera *Acidaminococcaceae*, *Streptococcus*, *Lactobacillus*, *Veillonella*, *Prevotella*, *Solobacterium*, *Scardovia*, and *Atopobium*. Some of these were associated with the increasing severity of WSLs such as *S. wiggsiae*, *S. salivarius* and *V. atypica* and might play important roles in the process of caries development. The *in vivo* model of microbial community succession provided novel insights into the microbial community shifts associated with the development of WSLs, and likely dental caries. Consequently, this model can be used to study the efficacy of treatment or perturbations, such as diet, antibiotics, and other prophylactic and restorative

treatments, and may help design more effective interventions and preventions.

Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors. Funding by the Faculty of Dentistry, University of Oslo, Oslo, Norway.

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***Ingar Olsen**
 Department of Oral Biology
 University of Oslo
 PB 1052 Blindern
 0316 Oslo
 Norway
 Tel: +47-22840350
 Fax: +47-22840302
 Email: ingar.olsen@odont.uio.no

